Defining the Mechanism of Action of Multi-Walled Carbon Nanotubes on Immune Cells *in Vitro*

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Tiivistelmä

Teollisesti tuotetut nanomateriaalit muodostavat nopeasti kasvavan joukon kemikaaleja, joiden käyttökohteet ovat lähes rajattomat. Näitä materiaaleja sovelletaan niin kosmetiikassa ja ruoassa kuin lääketieteessä sekä teollisissa prosesseissakin. Joidenkin nanomateriaalien tiedetään olevan ihmisille haitallisia. Suurin osa teollisesti tuotettujen nanomateriaalien haittavaikutuksista on liitetty niiden vuorovaikutukseen luonnollisen immuniteetin solujen kanssa. Tavallisimmin ihmiset altistuvat teollisesti tuotetuille nanomateriaaleille hengitysteiden kautta. Hengitysteistä nanomateriaalit saattavat siirtyä muualle elimistöön, sillä niiden pieni koko mahdollistaa ihmiskehon fysikaalisten esteiden läpäisyn. Toisaalta nanomateriaalit voivat myös aiheuttaa hengitysteissä tulehdusreaktion, jonka pitkittyminen saattaa johtaa krooniseen tulehdukseen sekä esimerkiksi fibroosiin. Nanomateriaalien tuotanto kasvaa jatkuvasti, ja uusia materiaaleja kehitetään nopeaan tahtiin, siispä niiden mahdolliset haittavaikutukset on selvitettävä nopeasti ja huolellisesti. Perinteinen riskiarviointi tapahtuu tavallisesti eläinkokeiden avulla ja keskittyy selvittämään altistukseen liittyviä riskejä sekä haittavaikutuksia, kun taas toksikogenomiikka puolestaan pyrkii kuvailemaan toksisuuden mekanismeja. Tässä tutkielmassa selvitetäänkin erään tuotetun nanomateriaalin, moniseinäisten hiilinanoputkien, transkriptionaalinen toimintamekanismi THP-1 monosyyteissä sekä PMA-erilaistetuissa makrofageissa DNA-mikrosirujen avulla. Solut altistettiin hiilinanoputkille 24, 48 ja 72 tunnin ajan kolmessa eri pitoisuudessa (5, 10 ja 20 µg/ml) annos-vastesuhteen määrittämiseksi. Tutkielman tuloksista päätellen monosyytit ja makrofagit reagoivat hiilinanoputkiin eri tavoin, makrofagien ollessa huomattavasti herkempiä altistukselle. Makrofageissa moniseinäiset hiilinanoputket aiheuttivat useiden immuunipuolustukseen liittyvien geenien sekä biologisten reittien yliekspressiota. Näistä päällimmäisinä esiin nousivat proinflammatoriset signalointireitit TNF- ja IL-17-signalointi. Proinflammatorinen vaste oli voimakkaimmillaan 24 tunnin altistuksen jälkeen, kun taas suoraa annos-vastesuhdetta osoittavien geenien osuus laski 48 tunnin ja 72 tunnin välillä, mikä viittaa muuttuneen mikroympäristön aiheuttamiin epäsuoriin muutoksiin transkriptomissa. Tämän tutkimuksen perusteella hiilinanoputkialtistus vaikutti suorasti NF-kappa B -signalointiin liittyvien geenien ekspressioon, nostaen kyseisen signalointireitin keskeiseen asemaan makrofagien vasteessa hiilinanoputkille. Tutkielman tulokset lisäävät ymmärrystä moniseinäisten hiilinanoputkien aiheuttamista molekulaarisista muutoksista luonnollisen immuniteetin soluissa.

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Abstract

Engineered nanomaterials (ENM) are a rapidly expanding class of chemicals with applications spanning from cosmetics to food, and from industrial applications to pharmaceuticals. Some ENM are already known to be harmful to humans. Majority of the adverse effects associated with ENM exposure are mediated by the cells of the innate immunity. The respiratory system offers a vulnerable route of exposure for ENM as they are small enough to pass through many of the physical barriers of the body and even translocate to sites beyond the point of entry. In the lungs, ENM tend to induce an inflammatory reaction that can turn chronic and lead to other conditions such as fibrosis. As the production of ENM is constantly increasing and novel materials are reaching the market in an increasing pace, their toxic potential must be well characterised. While the traditional hazard assessment is driven by apical endpoints and heavily focused on expensive and time-consuming animal experiments, toxicogenomics approaches turn the focus onto the mechanisms of toxicity. In this thesis, the transcriptional mechanism of action (tMOA) of multi-walled carbon nanotubes (MWCNT) was characterised in THP-1 monocytes and PMA-differentiated macrophages using DNA microarrays. The exposures were carried out for 24, 48 and 72 hours with three doses (5, 10 and 20 µg/ml), for determining the dose-response relationship with benchmark dose modelling. The results suggest distinct transcriptomic responses in monocytes and macrophages, with macrophages being significantly more sensitive. In macrophages, MWCNT induced the expression of a wide range of immune related genes and pathways, including proinflammatory signalling pathways such as TNF and IL-17 signalling. The proinflammatory response was more pronounced after 24 hours of exposure, while transcriptomic alterations probably mirroring indirect changes in the microenvironment increased between 48 hours and 72 hours, as suggested by the decreased proportion of dose-dependent genes at 72 hours. From this analysis, the genes associated to the NF-kappa B signalling pathway emerged as directly affected by the exposure, suggesting their role as key regulators of the macrophage response to MWCNT. These results contribute to a better understanding of the molecular alterations caused by MWCNT exposure on innate immune cells.

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Table of contents

1 **Introduction** 1

2 **Literature Review** 2

2.1 Engineered nanomaterials 2

2.1.1 Immunotoxicity 3

2.1.2 From nanotoxicology to nanopharmacology 4

2.2 Toxicology 6

2.2.1 Risk Assessment 6

2.2.2 Dose-response 7

2.2.3 Towards Modern Toxicology 8

2.2.4 Toxicogenomics 8

2.3 Transcriptomics 11

2.3.1 Gene expression profiling by DNA microarray 12

2.3.2 DNA Microarray data pre-processing 13

3 **Aims of the Study** 15

4 **Materials and Methods** 16

4.1 Nanomaterial characteristics 16

4.2 Differentiation and exposures 16

4.3 RNA extraction and quality check 17

4.4 Transcriptomics profiling 17

4.5 DNA microarray data analysis 18

4.6 Cell type comparison 18

4.7 Pathway enrichment analysis 19

4.8 Benchmark dose analysis 19

5 **Results** 20

5.1 PMA induces the expression of immune genes 20

5.2 MWCNT exposure exerts distinct patterns of transcriptomic alterations in monocytes and macrophages 22

5.3 NF-kappa B signalling pathway genes are altered in a dose-dependent manner 24

6 Discussion **26**

7 Conclusions **31**

8 References **32**

9 Appendices **38**

Abbreviations

AOP	Adverse outcome pathway
AO	Adverse outcome
BMD	Benchmark dose
BMR	Benchmark response
CNT	Carbon nanotubes
cDNA	Complementary deoxyribonucleic acid
DAMP	Damage-associated molecular pattern
ECHA	The European Chemicals Agency
ENM	Engineered nanomaterials
FBS	Foetal Bovine Serum
GI	Gastrointestinal
GO	Gene ontology
In silico	Performed on a computer
In vitro	Performed outside a living organism
In vivo	Performed in a living organism
KE	Key event
KEGG	Kyoto Encyclopedia of Genes and Genomes
MIE	Molecular initiating event
MOA	Mechanism of action
MWCNT	Multi-walled carbon nanotubes
NAMP	Nanoparticle-associated molecular patterns
NLR	NOD-like receptor
NF-κB	Nuclear factor kappa B
NOAEL	No-observed-adverse-effect-level
OECD	Organisation for Economic Cooperation and Development
PAMP	Pathogen-associated molecular pattern
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
RNA-seq	RNA sequencing

ROS	Reactive oxygen species
RQN	RNA quality number
RT-qPCR	Real-time quantitative polymerase chain reaction
THP-1	Human monocytic leukaemia cell line
TLR	Toll-like receptor
TNF	Tumour necrosis factor
tMOA	Transcriptional mechanism of action
TOX21	Toxicology in the 21st Century
3R	Reduction, refinement, and replacement of animal experiments

1 Introduction

Humans have learnt to manipulate matter on molecular, even atomic level, leading to innovations far beyond the imagination of our ancestors. A tour de force of such manipulation is an emerging class of substances called engineered nanomaterials (ENM). These nanoscale particles can be engineered to fit the ever-growing needs of today's society. They are already implemented in a range of consumer products, including cosmetics, clothes, toys, and sports equipment. They are employed in industrial applications, and even medical purposes. Although these tiny particles have the potential to change our lives in many aspects, concerns about their health effects are arising (Rydman et al. 2014; Kinaret et al. 2017b). The increasing use and production of ENM is contributing to the growing particle burden in our everyday lives, and it could be affecting our health. Indeed, many kinds of ENM have been found to interact with various immune cells potentially leading to immunotoxic effects (Farrera and Fadeel 2015).

To fully unleash the potential of ENM, their risks must first be well characterised. The rapidly increasing range of different ENM rushing to the market requires careful and systematic evaluation of possible health hazards caused by these particles. Traditional risk assessment is generally based on expensive, ethically questioned, and time-consuming animal experiments, with little focus on the mechanisms of toxicity. For this, toxicogenomics approaches have been used to clarify the mechanism of action (MOA) of substances (Serra et al. 2019). Transcriptomics technologies allow a look inside the biological system to explore the molecular alterations caused by substances. This information can be further utilised to evaluate the toxicity of such exposures, and, eventually, steer us into designing novel substances that could be safe and functional by design.

In this thesis, I explore the transcriptomic alterations in immune cells due to multi-walled carbon nanotube (MWCNT) exposure to characterise their transcriptional mechanism of action (tMOA) across time at several concentrations *in vitro*. I identify the perturbed biological pathways contributing to the inflammatory response initiated by the interactions between macrophages and the MWCNT. This thesis contributes to the mechanistic information of immunomodulatory effects of MWCNT.

2 Literature Review

2.1 Engineered nanomaterials

Nanotechnology is one of the Key Enabling Technologies in the European Union's Horizon 2020 programme. Undeniably, nanotechnology has enormous potential in multiple fields, from material science to medicine, electronics, and robotics. Engineered nanomaterials (ENM) are already implemented across these fields and further applications are currently under investigation. This class of substances is extremely diverse, however, all of them have something in common: their size. Engineered nanomaterials are minuscule, intentionally produced particles with at least one dimension measuring less than 100 nanometres, making them similar in size to some viral particles or even smaller. They can be manufactured in a variety of chemical compositions, shapes, and functionalisations (Figure 1).

Their size, shape and chemical composition together create the unique properties that have made ENM an attractive source for numerous applications. For example, silver nanoparticles are used for their antimicrobial properties, metal oxides are typical pigments in food, cosmetics, and paints, while carbon based nanomaterials are used for their strength, conductivity, and chemical inertness (Cha et al. 2013; Qing et al. 2018). Although ENM are already exploited in a range of industrial applications as well as numerous consumer products, the special properties of ENM also raise concerns about their effects on human health and the environment.

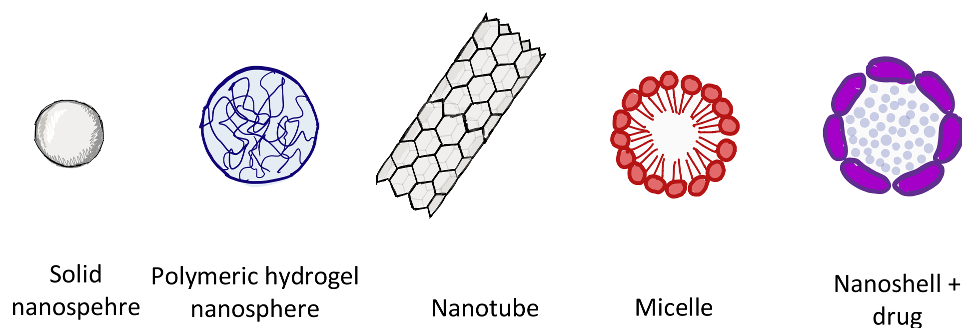

Figure 1. Schematic illustration of examples of different types of engineered nanomaterials. Size of the particles is not in scale. Adapted from Gubala et al. (2017).

Many ENM are able to pass through the epithelial barriers of the body and translocate to further tissues and organs (Czarny et al. 2014; Husain et al. 2015; Miller et al. 2017; Zhang et al. 2019). While their size partly accounts for the direct interactions with cellular structures and thus cell damage and oxidative stress, not all ENM are made equal. The chemical properties arising from

the core material as well as surface modifications affect the nano–bio interactions making some materials potentially more harmful than others. In addition to chemical properties, physical properties, such as the shape and the aspect ratio, are key players in the nano–bio interactions (Kinaret et al. 2017a).

Human exposure to these tiny particles happens mostly through the dermal route, by ingestion or through the respiratory system (Liu et al. 2017). Although the skin offers a great shield against most foreign agents, some ENM are small enough to pass through healthy skin, and even more if the condition of the skin is compromised (Larese Filon et al. 2015). In the gastrointestinal (GI) tract, ENM can interact with GI tissues as well as the intestinal microbiome (Bouwmeester et al. 2018). Dermal and oral exposures tend to be the most prominent for consumers as these particles are prevalent in many cosmetics and food products. However, exposure through the respiratory system is a serious concern particularly in occupational settings. Pulmonary exposure to nanomaterials is associated with adverse effects primarily mediated by the immune system, including chronic inflammation, allergic asthma, fibrosis and even increased risk of cardiovascular disease (Rydman et al. 2014; Poulsen et al. 2015; Kinaret et al. 2017b; Miller et al. 2017; Urbančič et al. 2018).

2.1.1 Immunotoxicity

The immune system has developed to protect us from numerous external and internal threats. The well-tuned interplay of different components of the immune system requires a balance of activation and inactivation whilst breaking this balance can lead to impaired immune responses. Immunotoxic effects can arise from both ends of this scale; overactivation can lead to chronic inflammation, autoimmunity, and hypersensitivity as well as tissue damage, while immunosuppression leads to inadequate protection against pathogens and tumour cells.

Constant contact with the outside environment makes the respiratory system extremely vulnerable to foreign threats. To protect us from pathogens and intruding particles, the lungs are equipped with an army of immune cells. While most ambient particles get trapped in the upper respiratory tract and are removed by the mucociliary escalator, many ENM are small enough to travel all the way to the deepest parts of the lungs (Geiser and Kreyling 2010). As the tiny nanoparticles reach their way to the alveoli, they are faced with residential macrophages. Macrophages aim to clear out the particles by ingesting them. If macrophages are not fully able to clear out the particles, or have trouble breaking them down, an

inflammatory reaction is initiated by the production of pro-inflammatory mediators such as cytokines and reactive oxygen species (ROS) (Farrera and Fadeel 2015). These mediators work as a signal to recruit other inflammatory cells, including granulocytes, to the site. The cytokines produced by macrophages also modify the environment, for instance by affecting the permeability of the vascular system in the area, allowing the immune cells to pass through more effectively (Maloney and Gao 2015).

As discussed above, the physicochemical characteristics of nanomaterials affect their interactions with biological systems, including the immune cells (Liu et al. 2017). While the surface characteristics can largely affect biomolecule adsorption and the direct interactions with membranes and other cellular structures, some of the most severe consequences of ENM exposure have been linked to high aspect ratio nanomaterials. More specifically, rigid multi-walled carbon nanotubes (MWCNT) have been extensively studied as their adverse effects have been compared to those of asbestos (Poland et al. 2008). MWCNT are cylinder-shaped nanoparticles formed from several one-atom-thick graphite sheets rolled up to form a tubular structure. They are reported to be very biopersistent, and some MWCNT are recognised as carcinogenic (Labib et al. 2016; Fukushima et al. 2018). Many studies have focused on more immediate effects of MWCNT exposure (Rydman et al. 2014; Poulsen et al. 2015; Kinaret 2017b), which are mainly initiated by the interactions between macrophages and the nanotubes.

The long and rigid structure of certain MWCNT makes it difficult for macrophages to fully engulf them, leading to a process referred to as frustrated phagocytosis (Rydman et al. 2014). Frustrated phagocytosis leads to further release of mediators recruiting monocytes and macrophages from the bloodstream. Multiple macrophages fuse together to form foreign body giant cells (Rydman et al. 2014). This impartial clearance of MWCNT can create prolonged inflammation and finally granulomas and fibrosis in the lung tissue (Rydman et al. 2014; Poulsen et al. 2015; Kinaret et al. 2017b).

2.1.2 From nanotoxicology to nanopharmacology

The same properties of the ENM that can make us wary of their negative health effects are also what has caught the attention of medical and pharmacological fields. Several types of ENM are used as agents in biomedical imaging (Han et al. 2019) and many are investigated for a great promise in tissue engineering applications (Shadjou et al. 2018). In terms of nanopharmaceuticals, many nano-enabled drugs are already on the market and a range of

clinical trials are ongoing (Weissig et al. 2014). To date, most nanopharmacological applications have been focused on nanoparticles as drug carriers and not so much as therapeutic agents themselves. However, recently the immunomodulatory properties of ENM have been gaining attention, suggesting novel therapeutic applications (Liu et al. 2018). These immunomodulatory properties have already been studied in the form of regulating antimicrobial immune responses as reported by (Gao et al. 2015). Furthermore, some ENM have been shown to affect macrophage polarisation, and thus promote specific types of immune responses. For example, (Zanganeh et al. 2016) described macrophage polarisation towards a pro-inflammatory phenotype with iron oxide nanoparticles leading to decreased tumour growth. MWCNT, on the other hand, seem to promote a mixed status of macrophage polarisation, as reported by (Meng et al. 2015) as well as Kinaret et al. (under revision). Thus, ENM hold enormous potential across multiple pharmacological applications. The safe translation of ENM into therapeutics, however, requires a thorough understanding of their toxicity at all levels of biological organisation.

2.2 Toxicology

Toxicology, the study of poisons, has existed as a scientific discipline for several hundreds of years, while poisonous substances have been a part of the human life through the evolution of our species. Toxicology dawned as a necessary subdiscipline of medicine and pharmacology until the explosion of the chemical industry in the late 19th century (Marquardt et al. 1999). Although still an essential part of the pharmacological field, toxicology has grown on its own to answer the demand created by the ever-growing chemical burden in our environment and everyday lives. The many branches of today's toxicology aim to assess, characterise and ameliorate the risks posed by chemical, physical and biological agents to human health and the environment.

2.2.1 Risk Assessment

Human health risk assessment is in the core of toxicology. It is a process that evaluates the potential adverse health effects caused by chemical and physical exposures, including particles. The US National Research Council set the framework for risk assessment in 1983 as four steps: i) hazard identification, ii) dose-response assessment, iii) exposure assessment, and iv) risk characterisation (National Research Council (US) Committee on the Institutional Means for Assessment of Risks to Public Health 1983). Similar protocol has been adopted by the European Chemicals Agency (ECHA) as guidelines for the health risk assessment required by the European Union's regulation REACH (ECHA, 2015). REACH stands for Registration, Evaluation, Authorisation and Restriction of Chemicals and it was established by EU in 2007 for the increased protection of human health and the environment. REACH controls the marketing and use of all chemical substances within the EU.

The systematic risk assessment protocol conducted under REACH calls for effects assessment, which requires both hazard identification as well as hazard characterisation. Hazard identification considers all possible adverse effects the substance might cause, including acute and repeated dose toxicity, irritation, corrosivity, sensitisation, mutagenicity and carcinogenicity as well as reproductive toxicity. Hazard characterisation, on the other hand, tackles the effects of the dose and the level of exposure, generally referred to as the dose-response. Details of the substance's toxicokinetic behaviour, including absorption, distribution, metabolism and excretion, should be provided when available, although the generation of such data is not required by REACH (ECHA, 2011). Following the effects assessment, the exposure

assessment includes characterisation of the physicochemical properties of the substance, the likely routes of exposure and exposure levels, the duration and frequency of a typical exposure, as well as description of the population susceptible to exposure. Lastly, the risk posed by the combination of the exposure and the hazard characterisation is considered. In a complete risk characterisation, it is essential to also acknowledge the exposure to other substances in combination to the one under assessment.

2.2.2 Dose-response

Dose-response assessment is one of the cornerstones of toxicological research. Already stated by Paracelsus, the man credited as the founder of modern toxicology, "the dose makes the poison" is now seen as one of the basic principles of toxicology (Borzelleca 2000). As a part of risk assessment, quantitative estimation is used to determine safe doses for substances. The direct effects of chemical and physical stressors are typically expected to increase with higher doses in a monotonic manner (Dorato and Engelhardt 2005), although non-monotonic dose-response curves for certain substances are also reported (Vandenberg 2014).

Several strategies for determining safe doses have been developed. In risk assessment, one accepted strategy is to determine the no-observed-adverse-effect-level (NOAEL), which is generally defined as the highest experimental dose in which no adverse effects are observed (Dorato and Engelhardt 2005). Although NOAEL is commonly used in regulatory toxicology, its definition is not as simple as it seems. Some problems associated with the use of NOAEL include the fact that it is limited to the experimental design as it takes into consideration only the doses used in the experiment. The obtained value is also dependent on the experimental model in use, the number of animals or samples, and simply the definition of the adverse effect (Lewis et al. 2002; Haber et al. 2018).

Another approach applied in risk assessment is benchmark dose (BMD) modelling. The estimation of the BMD is based on fitting a mathematical model to represent the dose-response and defining a dose or a concentration at which a specific, predefined level of response is estimated to be observed. This level is referred to as the benchmark response (BMR), and it is commonly defined either as one standard deviation or 10% above or below the background (Qutob et al. 2018). Unlike NOAEL, the BMD approach considers the whole dose response curve and thus allows the estimation of a BMD even below experimental doses (Haber et al. 2018). Furthermore, BMD modelling offers a better statistical power with a smaller number of

animals, makes use of all test points as well as allows comparison between different datasets (Slob 2014). For these reasons, BMD modelling has become the preferred method for exploring points of departure and determining safe limits for regulatory purposes (Haber et al. 2018).

2.2.3 Towards Modern Toxicology

The human health risk assessment strategy as well as other toxicological studies have traditionally been heavily based on animal experiments. This makes them expensive and time-consuming, while also raising concerns about ethical aspects and translatability. Several strategies have been initiated to modernise the field of toxicology. The 3R (Refine, Reduce, and Replace) principles for animal testing were developed over half a century ago, while more recently the use of alternative testing strategies has been promoted in the REACH as well as the USA based federal program TOX21. The enormous amount of new and untested substances requires a rapid development of faster and more affordable testing strategies, while pressure to move away from animal-based toxicology is also growing.

In vitro models for toxicity testing are gaining ground both inside and outside of regulatory toxicology. They allow the use of human cells and tissues while diminishing the number of animals needed for safety assessment. However, *in vitro* models alone tend to lack the complexity of real-life exposure scenarios and still do not allow a thorough evaluation of long-term exposures. *In silico* methods for toxicology are also being developed for analysing, visualising, and predicting the toxic potential of substances (Raies and Bajic 2016). Nonetheless, these alternative methods fail to answer the need for more effective safety assessment unless they are properly combined into standardised procedures.

2.2.4 Toxicogenomics

The concept of toxicogenomics emerged two decades ago and it has been gaining ground as a complementary strategy in toxicology since then (Nuwaysir et al. 1999). While traditional toxicology focuses on the evaluation of phenotypic changes *in vivo*, toxicogenomics aims to characterise the underlying mechanisms of toxicity by the use of omics technologies, such as transcriptomics, epigenomics, proteomics, and metabolomics (Wilmes et al. 2015; Kinaret et al. 2017a; Scala et al. 2018; Wolters et al. 2018). Toxicogenomics enables the needed shift from apical end-point focused approach to a system-wide and mechanistic view of toxicity, allowing a thorough understanding of molecular mechanisms leading to toxic outcomes.

In toxicology, the mechanism of action (MOA) can be defined as the set of molecular alterations in a cell or a tissue due to a specific exposure (Kinaret et al. 2017a; Serra et al. 2019). Thus, in the case of a transcriptomics based toxicogenomic study, defining the transcriptional mechanism of action (tMOA) refers to all of the transcriptomic alterations in the exposed system. These alterations can be further explored in the form of perturbed molecular pathways, processes, and networks, creating a more thorough picture of the mechanisms behind toxic outcomes. Toxicogenomics also has an important role in the adverse outcome pathway (AOP) framework initiated by the OECD in 2012. The concept of AOPs consist of a molecular initiating event (MIE) and subsequent key events (KE) at different levels of biological organisation, finally leading to an adverse outcome (AO). Toxicogenomic approaches can aid in recognising the MIE as well as the KE and relevant biological pathways whose perturbations lead to the AO (Labib et al. 2016; Brockmeier et al. 2017). A conceptual AOP for MWCNT exposure in the lung tissue is represented in Figure 2.

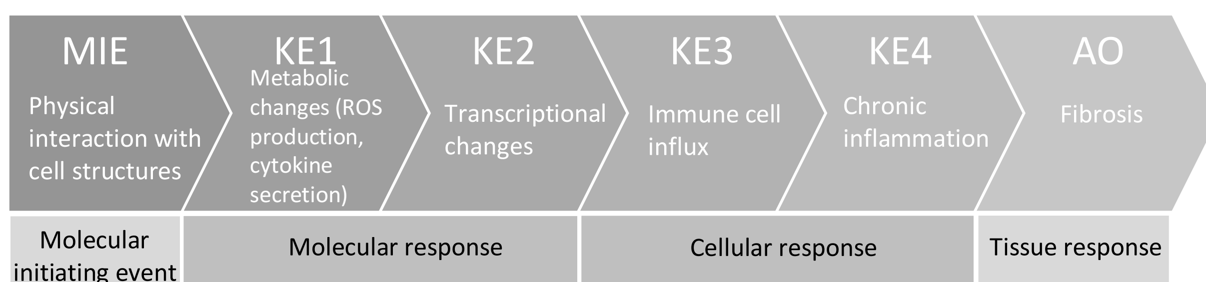

Figure 2. Conceptual adverse outcome pathway (AOP) for multi-walled carbon nanotube exposure in lung tissue.

The mechanistic information derived from toxicogenomic studies not only gives insight into why an exposure is toxic on a molecular level, but also allows to recognise toxic outcomes prior to apical endpoints being observed. For example, transcriptional BMD values obtained for relevant biological pathways have been shown to be comparable to apical BMD values (Labib et al. 2016; Qutob et al. 2018), thus proposing their use as points of departure for risk assessment (Thomas et al. 2013; Moffat et al. 2015).

Furthermore, toxicogenomics data has great potential for predicting compound toxicity (Blomme et al. 2009; Low et al. 2011) as well as for translating *in vitro* data into *in vivo* set ups (Kinaret et al. 2017a). The high-throughput approach to screening molecular alterations can also aid in the discovery of novel applications for existing compounds. Finally, understanding the relationship between the structure, activity, and the MOA of compounds allows for the

creation of new materials that could be safe-by-design. With these aspects in mind, toxicogenomics has the capability to speed up the risk assessment of compounds, including nanomaterials, while cutting back in animal experiments and costs.

2.3 Transcriptomics

The term transcriptome refers to all of the RNA transcripts present in a cell or tissue at a given moment. Omics technologies capture changes in gene expression and aid the characterisation of perturbed biological pathways and networks, thus providing mechanistic information behind observed phenotypes.

Gene expression can be studied in multiple ways. Current technologies for gene expression profiling are based either on hybridisation or synthesis and, based on their output volume, can be considered either low- or high-throughput. These technologies are summarised in Table 1. Available methods for large-scale transcriptomic profiling include DNA microarrays and RNA sequencing (RNA-Seq). Both microarrays and RNA-Seq are considered high-throughput, as they allow to measure a large number of transcripts simultaneously. Often these technologies are also referred to as high-content to better differentiate them from other high-throughput approaches in which a limited number of features are measured in a large number of biological samples in parallel. More targeted information about expressed genes and their expression levels can be obtained with commonly used low-throughput methods, such as northern blot and reverse transcriptase quantitative PCR (RT-qPCR). These low-throughput methods are limited to a small number of predefined targets, and with the increased availability of high-throughput methods they are mostly used for validating selected genes of interest (Poulsen et al. 2015; Alexander-Dann et al. 2018).

Table 1. Commonly used technologies to study gene expression.

	LOW THROUGHPUT	HIGH THROUGHPUT
HYBRIDIZATION	NORTHERN BLOT	MICROARRAY
SYNTHESIS	RT-qPCR	NGS

For the past two decades, DNA microarrays have been the prominent technology for large-scale gene expression profiling (Zhao et al. 2014). RNA sequencing has been gaining ground as a valid complementary approach over the last ten years. Results from the two technologies tend to have good correlation in terms of the overall gene expression profile, however, RNA-seq is generally more sensitive and able to capture genes expressed in very low levels (Zhao et al.

2014; Wolff et al. 2018). Microarray technologies, on the other hand, offer a well-established, quick, and more affordable option with more standardised and simple protocols of data analysis. Although the price gap between these transcriptomics technologies is getting smaller, microarray is still the preferred choice for gene expression profiling in toxicogenomics studies. Majority of already existing toxicogenomics data is conducted using microarrays and vast amounts of these data are available in public databases.

2.3.1 Gene expression profiling by DNA microarray

As DNA microarrays have been available for more than two decades, they are a fairly mature technology with well-established protocols. Several different gene expression DNA microarray platforms are commercially available. Regardless of the platform, DNA microarray technology is based on a chip containing predefined sequences of complementary DNA (cDNA), referred to as probes, with known locations on the chip. Depending on the application, these probes can cover the whole genome of the organism studied. RNA of interest is first reverse transcribed into cDNA that is then converted into cRNA labelled with a fluorescent marker. The labelled cRNA is hybridised with the probes, washed and scanned to measure the intensities emitted by the fluorescent labels. Studies comparing different microarray platforms show a good concordance, as long as the study is rigorously conducted (Irizarry et al. 2005; Petersen et al. 2005; Liu et al. 2012). Cross-platform comparison has also been improved with the advancement of data analysis methods as well as proper experimental design.

The design of the experiment is an essential step in every study. When planning a microarray experiment, it is crucial to recognise the factors that can affect the outcome of the experiment. In transcriptomics studies, the goal is often to identify genes that are differentially expressed between some groups. Ideally, the differences observed between the groups should be associated with the biological variables and not by technical variables such as batch, dye and the microarray slides themselves (Bryant et al. 2011). Taking these technical variables into account during the planning of the experiment will produce more reliable and reproducible results. Improper study design can result in confounded variables, which means that it is impossible to recognize to which extent the observed variability is due to each of the variables.

2.3.2 DNA Microarray data pre-processing

Once the data has been produced, it requires processing to ensure fair comparisons between samples. The standard workflow for microarray data processing has been established, and multiple computational tools have been developed to aid in the process. The best practice workflow for microarray data analysis is provided in Figure 3 (Marwah et al. 2019).

The raw data are complemented with the phenotypic data containing sample information including biological and technical variables. Quality of the data is assessed to identify apparent outliers and to ensure spot and hybridisation quality (Kauffmann et al. 2009). Probe quality is established by comparing the probe signals against negative control probes and poor quality probes are filtered out (Marwah et al. 2019).

For relevant comparisons between samples, expression values need to be normalised to the same scale. Normalisation is applied to eliminate systematic effects caused by technical differences (Ritchie et al. 2015). After normalisation, the effect of different variables on sample variation can be assessed. Ideally, observed variability should be associated mainly with biological variables. If systematic error due to technical variables is recognised, batch effect correction can be applied (Lazar et al. 2013). DNA probes on the microarray slide are annotated to known transcripts. Multiple probes mapped to the same transcript are combined to obtain single expression values for each gene or transcript. Pre-processed gene expression data can be further analysed. A key step is to identify differentially expressed genes between sample groups. Several statistical strategies can be applied, but a typical approach is to calculate fold changes and their statistical significance by comparing different sample conditions (Ritchie et al. 2015).

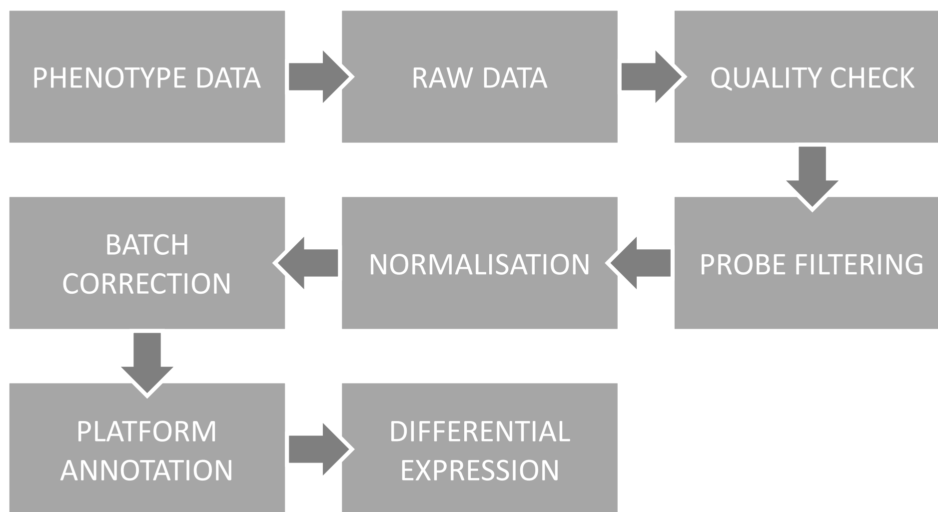

Figure 3. Best practice workflow for DNA microarray data preprocessing and analysis. Adapted from Marwah et al. (2019).

3 Aims of the Study

The aim of this study is to clarify the transcriptional mechanism of action (tMOA) of multi-walled carbon nanotubes (MWCNT) on monocytes and macrophage-like cells *in vitro*. The transcriptomic alterations are explored at multiple doses and time points to characterise the dose-response relationship as well as changes in the response across time.

4 Materials and Methods

4.1 Nanomaterial characteristics

Multi-walled carbon nanotubes used in the experiment have previously been characterised by Rydman et al. (2014) as well as Kinaret et al. (2017a). Material properties are listed in Table 2.

Table 2. Properties of the nanomaterial used in the experiment.

NANOMATERIAL PROPERTIES

Description	Rigid multi-walled carbon nanotube
Acronym	MWCNT
Product code	MWCNT-7 mitsui
Provider	Mitsui & Co., Ltd. (Japan)
Shape	Tube
Aspect ratio	2.6
Average length (nm)	13 000
Average diameter (nm)	50
Avg. surface area (m^2/g)	22

4.2 Differentiation and exposures

THP-1 cells (ATCC TIB-202) cultured in flasks in RPMI media that was supplemented with 10% FBS, 1% penicillin-streptomycin antibiotics and 2 mM ultraglutamine. To differentiate THP-1 cells into macrophage-like cells, the cells were distributed to six-well plates (1,000,000 cells/well) and subjected to 50 nM PMA (phorbol 12-myristate 13-acetate) for 48 hours. A stock solution of 1 mg/ml of MWCNT was made to complete RPMI media. The stock solution was vortexed and then sonicated for 20 minutes in a bath sonicator (Elmasonic S15H, Ilabequipment, USA). The stock solution was diluted to concentrations of 5, 10 and 20 µg/ml with the same media and vortexed and sonicated again for 20 minutes. Exposures were done as triplicates for both THP-1 monocytes and differentiated THP-1 cells by adding the final solutions on top of the cells on the six well plates. Control samples were left untreated for both cell types. Cells were harvested after 24h, 48h or 72h of exposure. The treatments are summarised in Figure 4.

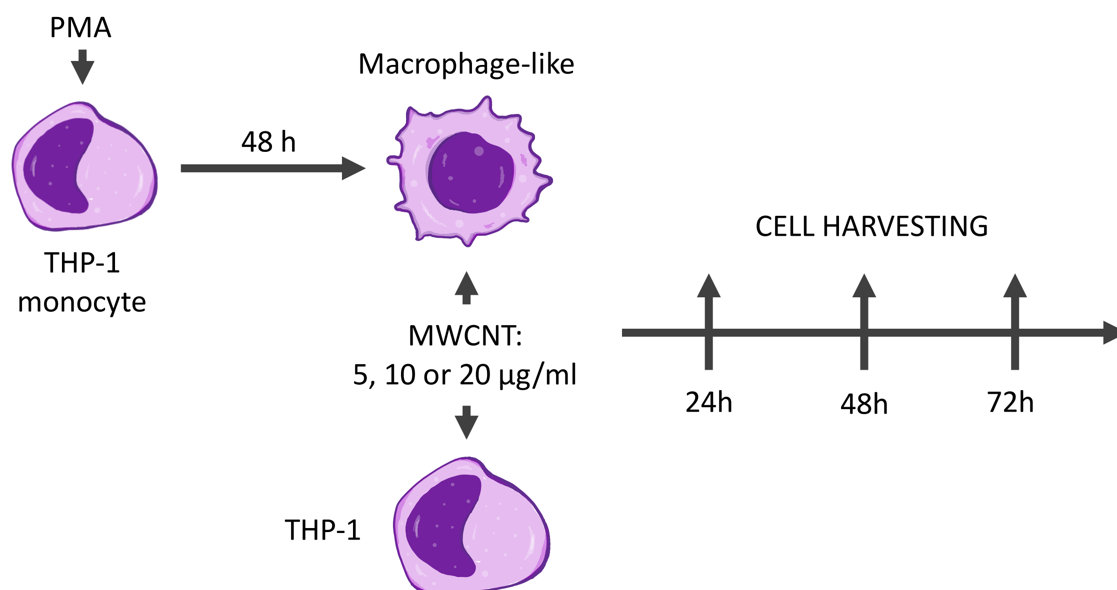

Figure 4. Experimental design of cell differentiation and exposure.

4.3 RNA extraction and quality check

After each exposure period, cells were harvested and lysed. Total RNA was extracted and purified using RNeasy Mini kit (Qiagen, Germany) according to the protocol provided by the manufacturer. The purity and yield of RNA was verified with NanoDrop (ND-1000, Thermo Fisher Scientific Inc., USA) and the quality was further assessed with FragmentAnalyzer (Agilent, USA). Samples with RNA quality number (RQN) higher than 8.1 were used for microarray analysis.

4.4 Transcriptomics profiling

Transcriptomics profiling was conducted using Agilent SurePrint G3 Human GE 8x60 DNA microarrays following the manufacturer's recommendations (Quick Amp Labeling, Agilent, USA). Shortly, 100 ng of total RNA was retro-transcribed into double stranded cDNA and further transcribed into cRNA and labelled using nucleotides containing either Cy3 or Cy5 fluorescent labels (Agilent, USA). cRNA was purified with RNeasy Mini Kit (Qiagen, Germany) and checked for quantity and specific activity with NanoDrop (ND-2000, Thermo Fisher Scientific Inc., USA). Two samples with different labels were combined and hybridised to the microarray slides. After 17 hours of hybridisation, the slides were washed and scanned with Agilent microarray scanner model G2505C. Data was extracted with Agilent Feature Extraction software version 12.0.2.2.

4.5 DNA microarray data analysis

Microarray data preprocessing and analysis were performed with the R Shiny application eUTOPIA (Marwah et al. 2019). Data for both cell types were analysed separately. Raw data files were uploaded together with phenotypic data containing both technical and biological variables. Quality check was performed, and low-quality probes were filtered out by selecting only probes with values higher than 75% quantile of negative control probes in at least 85% of the samples. Data were normalised between arrays using quantile normalisation method. Batch correction was performed using the method ComBat from R package sva (Leek et al. 2012) to eliminate the technical variation caused by the dye, slide, and row for macrophage-like cells, and dye, slide, and array for THP-1 monocytes, respectively. Probes mapped to the same gene symbol were summarised by their median values. Finally, differential expression analysis was performed by linear models followed by empirical Bayes pairwise comparisons, as implemented in the R package limma (Ritchie et al. 2015) approach. The variable of interest was specified as the combination of dose and time, and corrected batches were selected as covariates for the analysis. Differentially expressed genes were determined by comparing each group consisting of a specific dose and time point to the corresponding control group. Results were filtered by absolute log2 fold change of 0.58 and Benjamini & Hochberg adjusted p-value of 0.05.

4.6 Cell type comparison

The effect of PMA was studied by comparing the transcriptomic profiles of the two cell types in the untreated control samples. The genes in the separate expression matrices were first matched by names and the expression values were standardised using the R base function "scale". The scaled expression values were used for differential expression analysis with R package limma (Ritchie et al. 2015) as described above. In this case, the pairwise comparisons were done between macrophage-like cells and monocytes, for each time point separately. Finally, the results were filtered by absolute logFC of 0.58 and Benjamini & Hochberg adjusted p-value of 0.05 to obtain significantly differentially expressed genes between the cell types.

4.7 Pathway enrichment analysis

Pathway enrichments were conducted using the R shiny application FunMappOne (Scala et al. 2019). Gene symbols and their fold changes or BMD values at each experimental condition were used as an input. Functional annotation was specified as KEGG or Reactome, and only annotated genes were considered in the analysis. P-value threshold for pathway enrichment was set as 0.05 after FDR correction. Aggregation function was specified as "mean" and the final gene modification values were plotted based on their sign.

4.8 Benchmark dose analysis

For each time point, the log2 transformed data were analysed using a one-way ANOVA to identify genes that show a statistically different behaviour at different doses. The p-values were adjusted by using the FDR method. Only genes with an adjusted p-value lower than 0.01 were retrieved for further analyses.

For each of the previously selected genes, BMD analysis was performed by the following steps: first, a BMR value was identified as the expression value in the treated samples, giving at least 10% difference from the expression value of the corresponding controls; second, different matemathical models (linear, second and third order polynomial, exponential model, asymptotic regression model and Michaelis-Menten model) were fitted for every gene and used to predict the BMD values corresponding to the selected BMR; third, models with predicted BMD value greater than the maximum dose (20 µg/ml) or with a goodness-of-fit p-value lower than 0.1, were discarded. Among the remaining models, the optimal one is selected as the one with the lowest Akaike Information Criterion (AIC) value. (Serra et al. in prep.)

5 Results

In this study, I explored the transcriptomic effects of multi-walled carbon nanotube (MWCNT) exposure on immune cells *in vitro*. Multiple doses and time points were included to characterise the effect of dose and time.

5.1 PMA induces the expression of immune genes

First, I wanted to clarify the transcriptomic differences between the two cell types used in the experiment without considering the effect of the MWCNT exposure. Phorbol 13-myristate 12-acetate (PMA) changes the morphology of THP-1 cells. While THP-1 cells grow in a suspension, macrophage adhere to the bottom of the dish and obtain an irregular shape due to the formation of pseudopodia (data not shown). To characterise the difference in the transcriptomes of THP-1 monocytes and PMA-differentiated THP-1 macrophages, differentially expressed genes due to PMA-differentiation were explored in the control samples. The total of 1865 genes were differentially expressed at 24 hours, while a slight decrease in the number was observed at later time points with 1511 differentially expressed genes at 48 hours and 1585 genes at 72 hours, respectively (Figure 5A). Looking at the intersection of differentially expressed genes at all time points, 995 genes were shared between them.

Differentially expressed genes were further explored by pathway enrichment analysis. Selected KEGG pathways from the enrichment analysis are shown in Figure 5B. Immune system related genes were over-expressed in macrophages, while cell cycle related genes were expressed at higher levels in monocytes. Over-expression of genes related to lysosome and phagosome was also observed in macrophages. Similar results were seen when investigating the Reactome pathways, however, all cell cycle -related pathways were found not to be significantly enriched at 72 hours (Supplementary figure 1).

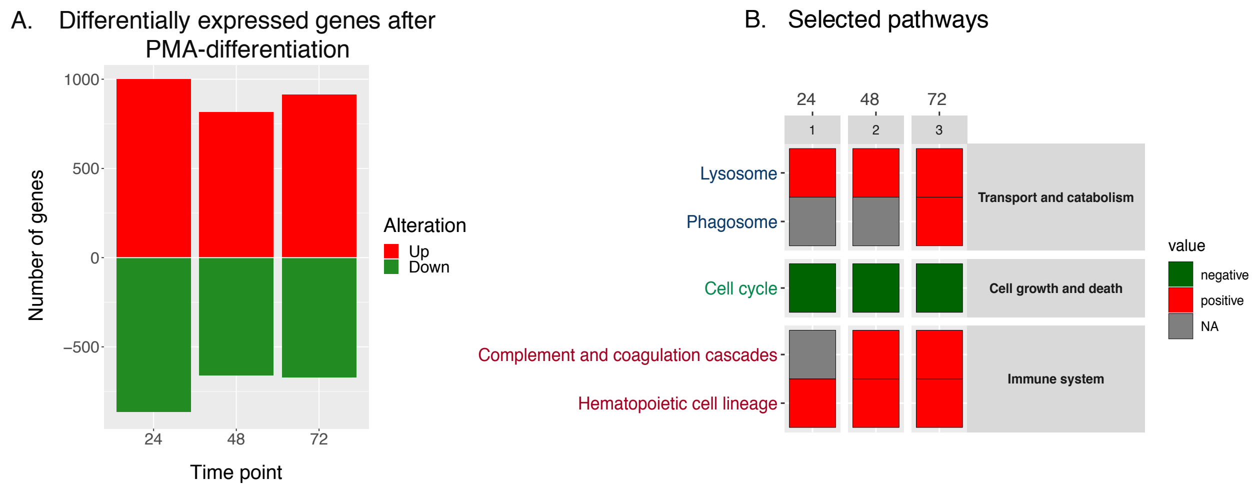

Figure 5 A. The number of differentially expressed genes in the control samples of each cell type at each time point. Red marks the genes that were upregulated after differentiation while green stands for downregulated genes. B. Selected enriched pathways as a result of PMA-differentiation of THP-1 cells.

The 995 genes at the intersection of all the time points were considered a stable set of genes that are altered in THP-1 cells undergoing PMA-differentiation. I compared this set of genes with tissue specific gene signatures available at the ARCHS4 database (Lachmann et al. 2018). This analysis resulted in significant enrichment of cell types "macrophage" and "alveolar macrophage" (adj. p-value 7.5e-80 and 1.3e-20, respectively).

I further focused on macrophage-specific marker genes, such as CD11B (gene also known as ITGAM), CD163 and IL1B. CD11B is known to be highly expressed in macrophages and dendritic cells (Ling et al. 2014), while CD163 is exclusively expressed in monocytes and macrophages, with higher expression in many tissue macrophages (Etzerodt and Moestrup 2013). IL1B codes for proinflammatory cytokine interleukin-1β, a typical marker of macrophage activation (Garlanda et al. 2013). Standardised mean expression values of these three genes in both cell types are represented in Figure 6. All selected genes were found to be significantly over-expressed in macrophage-like cells as a result of PMA-differentiation. However, a slight downwards trend with time can be observed.

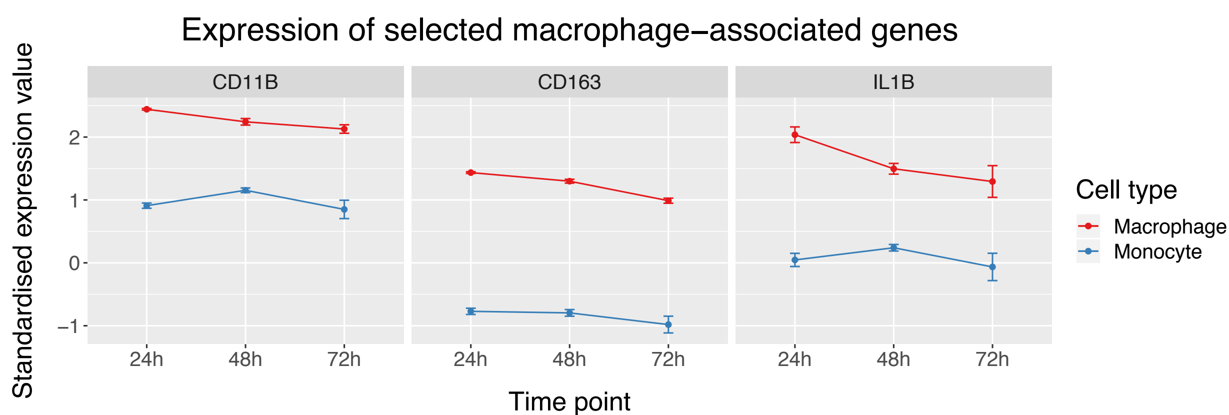

Figure 6. Standardised expression values of selected macrophage-associated genes in the untreated samples for both cell types.

These results confirm the morphological changes induced by PMA-differentiation also at the transcriptomic level.

5.2 MWCNT exposure exerts distinct patterns of transcriptomic alterations in monocytes and macrophages

The transcriptomic alterations due to MWCNT exposure were explored in human monocytic cell line THP-1 as well as in PMA-differentiated macrophages derived from THP-1 cells. Both cell types were exposed to three doses of 5, 10 and 20 µg/ml of MWCNT for three different exposure times: 24, 48 and 72 hours to investigate the effect of time and dose. In monocytes, the number of differentially expressed genes ranged from 0 to 12, except at the highest dose at 72 hours, where a total of 141 genes were found to be differentially expressed (Figure 7A). Out of these 141 genes, the expression of 102 genes was downregulated in the MWCNT exposed cells compared to untreated controls.

In macrophages, the total number of differentially expressed genes ranged from 575 at the lowest dose at 24 hours to 4,334 at 20 µg/ml at 48 hours (Figure 7B). At each time point, a dose-response relationship can be seen, as the number of differentially expressed genes increases with dose. The total number of differentially expressed genes peaked at 48 hours at each dose. The proportions of upregulated and downregulated genes were fairly equal in all conditions tested.

Figure 7 A. Number of differentially expressed genes in THP-1 monocytes due to MWCNT exposure. B. Differentially expressed genes in PMA-differentiated macrophages at each dose and time point.

The pathway enrichment for all conditions in both cell types was performed. For monocytes, using a nominal p-value of 0.05 for KEGG pathway enrichment resulted in only one enriched pathway in one condition (data not shown). However, with Reactome, five significantly enriched pathways were retrieved as represented in Figure 8.

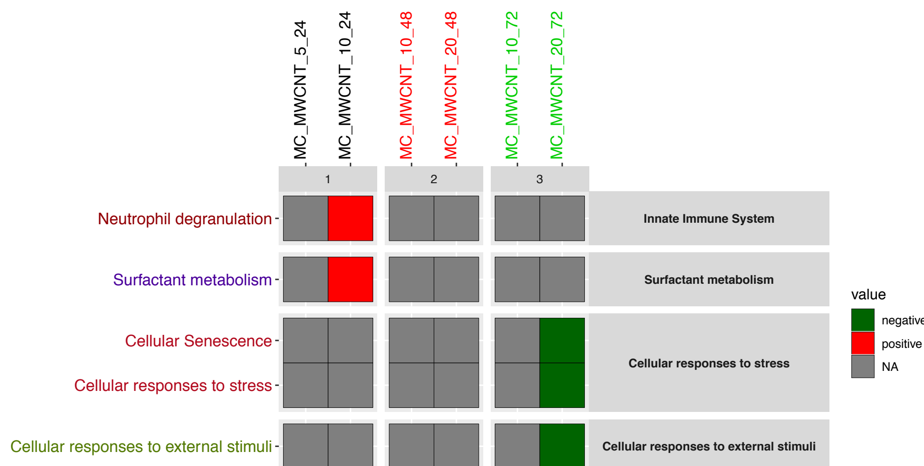

Figure 8: Reactome pathway enrichment map generated for differentially expressed genes in THP-1 monocytes at each dose and time point.

In macrophages, I retrieved a total of 86 KEGG pathways to be over-represented in one or more sets of differentially expressed genes (Supp. 2). I further selected significant pathways based on their pattern across doses and time points (Figure 9). This analysis resulted in 16 pathways, mainly related to immune functions. Narrowing down the number of differentially expressed

genes to top 100 upregulated and downregulated genes based on the fold change resulted in the enrichment of 28 KEGG pathways (Supp. 3).

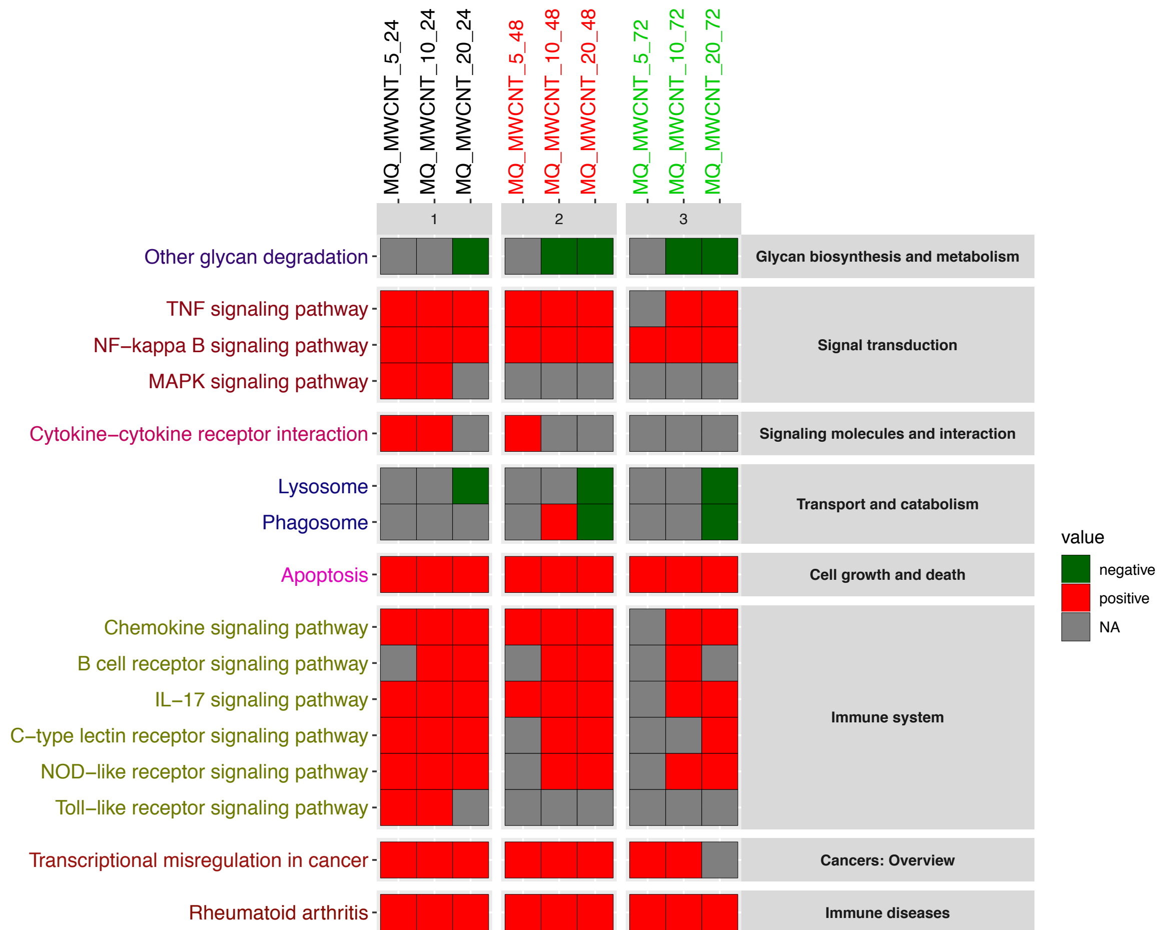

Figure 9. Selected significantly over-represented KEGG pathways retrieved for sets of differentially expressed genes in each experimental condition in macrophages.

5.3 NF-kappa B signalling pathway genes are altered in a dose-dependent manner

Finally, I wanted to distinguish the genes that were directly affected by the exposure from those that were indirectly affected (autocrine/paracrine signalling, changes in the microenvironment) in macrophages. With benchmark dose modelling, I was able to retrieve genes whose expression was altered dose-dependently in a monotonic manner (Figure 10A). As many as 1,001 were found to be dose-dependent at 24 hours, 1,838 at 48 hours, and 1,012 at 72 hours, respectively. Moreover, the sets of dose-dependent genes at each time point resulted in two enriched KEGG pathways at 48 hours: NF-kappa B signalling pathway and osteoclast differentiation. Genes annotated to NF-kappa B signalling pathway are shown in Figure 10B.

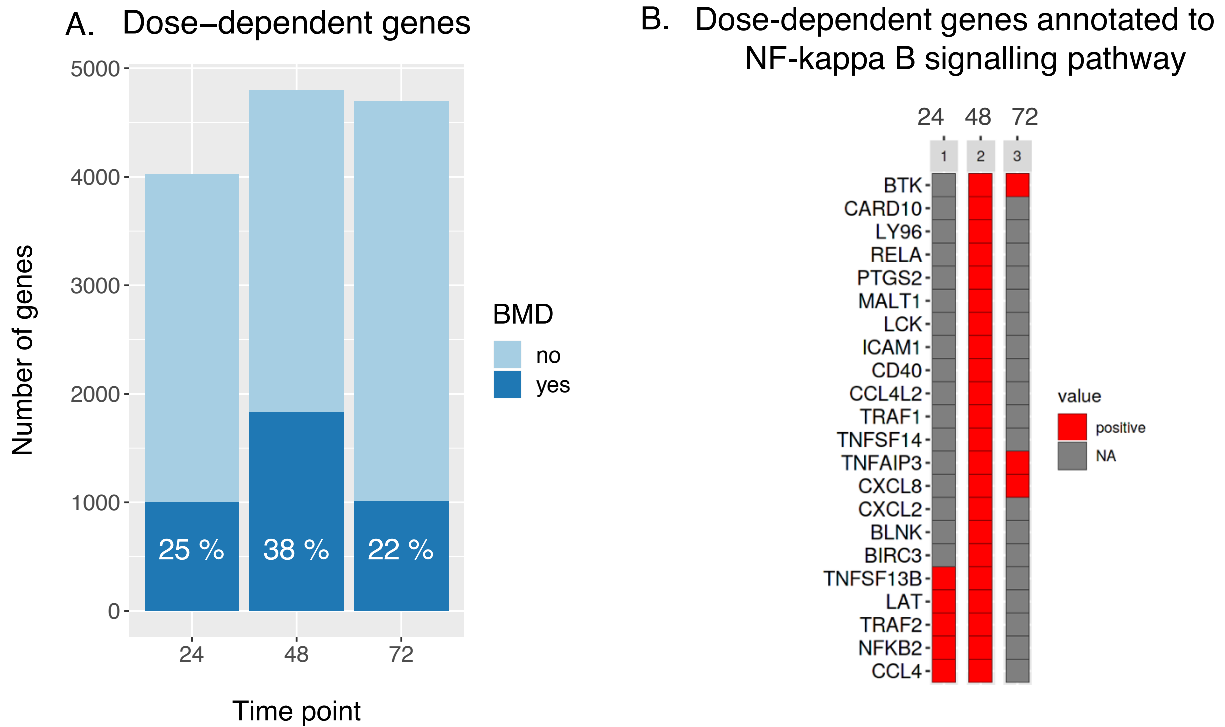

Figure 10 A. Proportions of monotonically dose-dependent genes at each time point relative to the union of all differentially expressed genes at said time point. B. Dose-dependent genes contributing to the enrichment of NF-kappa B signalling pathway in macrophages.

As many as 78 (2.1% of all the genes determined dose-dependent) genes were common to all time points. The unique genes for each time point accounted for 16.2% (24 hours), 36.6% (48 hours) and 21.4% (72 hours) of the total number of dose-dependent genes, respectively (Figure 11).

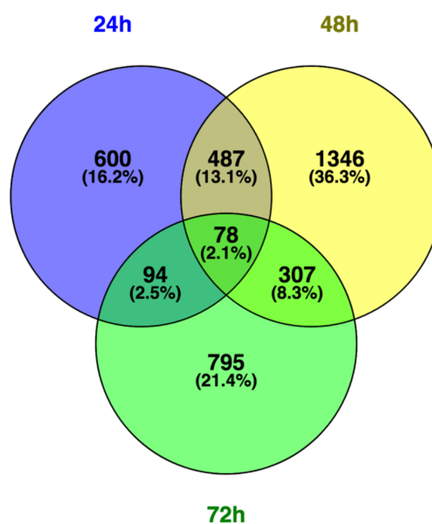

Figure 11. Venn diagram representing the proportions of monotonically dose-dependent genes at each time point.

6 Discussion

In this thesis, I characterised the transcriptional mechanism on action (tMOA) of one type of long and rigid multi-walled carbon nanotubes on immune cells *in vitro.* Multiple doses and exposure times were screened to evaluate the effect of the dose and to characterise the changes over time.

Macrophages are among the first cell types to interact with ENM in real-life exposure scenarios (Farrera and Fadeel 2015). Many of the adverse effects linked to ENM exposure are mediated by the interactions between macrophages and the nanoparticles (Dong et al. 2018). Monocytes, on the other hand, are precursor cells to macrophages and dendritic cells, while also expressing phagocytic activities. In this study, THP-1 monocytes and PMA-differentiated macrophages derived from them were used as models of immune cells. THP-1 is a human monocytic cell line that is derived from an acute monocytic leukaemia patient. It has been extensively studied and used as a model of human monocytes for its high degree of similarity with peripheral blood derived monocytes (e.g. Gao et al. 2000; Schroecksnadel et al. 2012).

Likewise, PMA-differentiated macrophages are a well-established model for studying macrophage functions. The morphological and transcriptional changes induced in THP-1 cells by PMA results in macrophage-like cells that have increased phagocytic activities, decreased proliferation and are adherent (Starr et al. 2018). Such changes were also observed in the current study on a transcriptomic level. As reported in Figure 5A and 5B, I observed major changes in the transcriptomic profile of PMA-differentiated cells versus THP-1 monocytes. As expected, pathway enrichment with these genes also confirmed decreased expression of genes related to cell cycle, while immune system related genes were expressed in higher levels in the differentiated cells.

Many genes associated with macrophages were expressed in higher levels in PMA-differentiated macrophages than THP-1 monocytes (Figure 6). Although these genes were found significantly differentially expressed at each time point, a slight decrease in their expression in time could be observed. A loss of enrichment of pathways related to the cell cycle was also observed in enrichment analysis for Reactome pathways, suggesting that less genes involved in these pathways were differentially expressed at later time points (Supp 1). However, for KEGG pathways, this loss was not observed (Fig. 5B). These results suggest that the effect

of PMA slightly decreases after removal of PMA from the growth media, yet no evidence in literature was found to explicitly support this. The decreasing expression could also be due to cell death, as the media was not changed during the treatment period. Nonetheless, both cell types have been extensively used in studying immune cell functions, and no major decrease in the confluence was observed during cell culture. PMA-differentiated macrophages also retained the morphological changes throughout the experiment.

The transcriptomic responses to MWCNT exposure were drastically different between monocytes and macrophages (Figure 7). Macrophages are known to phagocytise particles more efficiently than monocytes, thus supporting a more pronounced response in macrophages. THP-1 monocytes are also cultured in a suspension while PMA-differentiated macrophages are adherent. This difference in the culture conditions could affect the interactions between the cells and the MWCNT in the media, resulting in less physical contact between monocytes and the nanomaterial.

Monocyte response does not show a clear effect of the dose, as the number of differentially expressed genes is not increasing with the dose at the first two time points (Figure 7A). A higher level of differentially expressed genes, especially those that were downregulated at 72 hours and 20 µg/ml could indicate cell death. Downregulated genes were related to cellular responses to stress and external stimulation (Figure 8), suggesting impairment of these processes due to the exposure. Öner et al. (2017) have previously studied the cytotoxicity of MWCNT on THP-1 monocytes and reported no significant cytotoxicity at similar concentrations during a 24-hour exposure period. However, DNA damage due to MWCNT was observed at the dose of 25 µg/ml after 24 hours (Öner et al. 2017), which could eventually lead to impaired cell viability after a longer exposure time. Decreased monocyte viability has also been reported already after 24 hours of MWCNT exposure with the concentration of 25 µg/ml, thus cytotoxicity at such concentration cannot be ruled out (De Nicola et al. 2009).

Macrophages, on the other hand, exhibit major transcriptomic alterations due to MWCNT exposure. The number of differentially expressed genes also increased systematically with higher MWCNT concentrations (Figure 7B). Despite the fairly equal numbers of upregulated and downregulated genes, majority of enriched pathways were found among the upregulated genes, suggesting that MWCNT induce, rather than suppress, biological processes as their tMOA.

An interesting effect of the dose and time can be observed with multiple immune system - related pathways. For example, NOD-like receptor signalling pathway as well as C-type lectin receptor signalling pathway were found upregulated at 24 hours at each dose, while the effect seems to be fading over time for lower doses (Figure 9). Both NOD-like receptors (NLRs) and C-type lectin receptors are pattern recognition receptors (PRRs) that are crucial in the induction of an immune response to pathogens (Hoving et al. 2014; Kim et al. 2016). NLRs are intracellular receptors capable of recognising pathogen-associated molecular patterns (PAMP) as well as damage-associated molecular patterns (DAMPs). NLRs are also associated with recognising environmental particles and responding to cytokines, while also regulating apoptosis (Kim et al. 2016). Although nanoparticles have been proposed to be recognised by phagocytes as nanoparticle-associated molecular patterns (NAMPs) (Fadeel 2012), little evidence exists so far. Mukherjee et al. (2018) recently suggested that macrophages sense carbon nanotubes (CNT) *via* another PRR, Toll-like receptors (TLR). Indeed, in this study the TLR signalling pathway associated genes were observed to be upregulated in macrophages after 24 hours of exposure (Figure 9). Transcriptional upregulation of receptor signalling pathways, however, does not prove direct receptor interaction. Whether the enrichment of these pathways is due to interaction between the receptors and the MWCNT, due to DAMPs, or to the induction of downstream signalling molecules as part of the transcriptional response, requires further investigation. Upregulation of NLR signalling pathway, as well as TLR signalling pathway, upon CNT exposure has been reported in previous transcriptomic studies both *in vivo* and *in vitro,* proposing a systematic alteration of genes with similar functions across studies (Kinaret et al. 2017b; Mukherjee et al. 2018; Scala et al. 2018).

Similarly, TNF (tumour necrosis factor) signalling pathway as well as IL-17 signalling pathways lose their enrichment at the lowest dose at 72 hours (Figure 9). TNF and IL-17 are both proinflammatory cytokines, thus suggesting a quicker adaptation of the biological system at a lower dose. Chemokine signalling pathway had a similar pattern of enrichment (Figure 9). Interestingly, focusing on the top 100 upregulated and downregulated genes showed enrichment of proinflammatory pathways and receptor signalling pathways primarily at 24 hours, highlighting their importance in the regulation of the early immune response (Supp. 3). These transcriptional changes are confirmed in *in vivo* studies characterising the immune response in the lung (Poulsen et al. 2015; Kinaret et al. 2017b).

Nuclear factor-kappa B (NF-κB) signalling pathway was found enriched in macrophages in all the experimental conditions tested (Figure 9), besides being also one of the two significantly enriched dose-dependent pathways. NF-κB is a family of transcription factors regulating an array of genes involved in immune functions and inflammatory responses but also apoptosis and differentiation (Liu et al. 2017). Canonical NF-κB signalling pathway can be activated by various stimuli such as proinflammatory cytokines or through PRRs, while noncanonical pathway responds to TNF receptor superfamily members, including CD40 (Lawrence 2009; Liu et al. 2017), which is one of the dose-dependent genes contributing to the enrichment of NF-κB signalling pathway here (Figure 10B). The results of my analysis suggest NF-κB signalling pathway as a key player of the macrophage response to MWCNT. Indeed, NF-κB is known to have an important role in macrophage activation (Zhang et al. 2014). Furthermore, NF-κB signalling pathway has previously been suggested as a central regulator of transcriptional responses to CNT (Mukherjee et al. 2018). Specifically, NF-κB family member RELA was identified as a key gene in the regulation of macrophage activation (Zhang et al. 2014, Mukherjee et al. 2018, Zhao et al. 2018). In the current study, RELA was found upregulated at all time points, but dose-dependently only at 48 hours (Figure 10B).

Dose-dependently altered genes are typically considered as those that are directly affected by the exposure (Dorato and Engelhardt 2005). Here, BMD modelling was used to identify such genes among all differentially expressed genes. The selected mathematical models used in the analysis are accepted for BMD modelling for regulatory purposes (Serra et al. in prep), however, they only consider monotonically dose-dependent genes. If higher exposure concentrations affect cell viability, a decreased number of cells can result in lower expression levels, thus leaving these genes out of consideration. Cell viability and cytotoxicity assays could be used to rule out the effect of cell death in the observed expression levels. The concentrations used in the current study were fairly low and have previously been reported not to cause significant cell death (Scala et al. 2018). However, apoptosis related genes were found constantly upregulated in macrophages as a result of MWCNT exposure, indicating some degree of cellular stress at all tested concentrations (Figure 9). KEGG pathway “transcriptional misregulation in cancer” was likewise enriched at all time points. Indeed, MWCNT are recognised as potentially carcinogenic (Fukushima et al. 2018) and known to affect cell viability in higher concentrations (Scala et al. 2018).

Although NF-κB signalling pathway seems to be in the core of the macrophage response to MWCNT, a wide range of other genes were also dose-dependently altered as a result of the exposures. The proportion of dose-dependent genes out of all differentially expressed genes changes across time, suggesting a dynamic combination of direct and indirect effects (Figure 10A). At 24 hours 25% of differentially expressed genes are altered in a dose-dependent manner, and genes associated with proinflammatory pathways were among the most up-regulated, as discovered in the pathway enrichment (Supp. 3). These changes represent the initial response that triggers further molecular alterations. By 48 hours, the proportion of dose-dependent genes increased to nearly 40%, implying that the direct effect of MWCNT peaks after 48 hours of exposure, after which the contribution of indirect effects, such as changes in the microenvironment, could become more evident. The total number of differentially expressed genes remains nearly equal between 48 and 72 hours, while the proportion of dose-dependent genes drops close to 20% at 72 hours (Figure 10A). This could indicate adaptation in the form of transcriptomic alterations as well as responses to microenvironmental changes. The changing microenvironment is caused by molecules secreted by macrophages, such as cytokines. The macrophages themselves then respond to these changes, for instance by altering their polarisation status, as indicated by the recent findings of my colleagues (Kinaret et al. under review).

Transcriptomic changes only represent one layer of molecular alterations in a biological system. Whether the increased gene expression results in a production of a protein should be further investigated to confirm the effect of the changing microenvironment. Furthermore, in real-life exposures, macrophages and monocytes represent only a portion of the cell types encountered by MWCNT particles. Macrophages, however, are considered the initiating cells of the immune response, and thus, of most of the adverse effects associated with ENM exposures (Dong et al. 2018). Understanding the MOA on macrophages will shed light on the immunotoxic potential of MWCNT. To date, little information of ENM exposures on specific cell types is available. Furthermore, based on the response of the macrophages, it is possible to forecast the long-term effects of chronic exposures, such as the development of chronic inflammation, fibrosis or asthma, as a result of pulmonary exposures. Finally, modulating macrophage functions is emerging as a viable strategy in the development of immunotherapy (Cassetta and Kitamura 2018). Understanding the effects of engineered nanomaterials may help envisioning new therapeutic uses for them.

7 Conclusions

In this thesis, I characterised the transcriptional mechanism of action of multi-walled carbon nanotubes on monocytes and macrophages. The two cell types exhibit distinct patterns of transcriptomic alteration as a result of MWCNT exposure, with more pronounced changes observed in macrophages. MWCNT induce the expression of immune related genes and pathways in macrophages. Genes involved in the NF-kappa B signalling pathway emerged as key regulators of the response.

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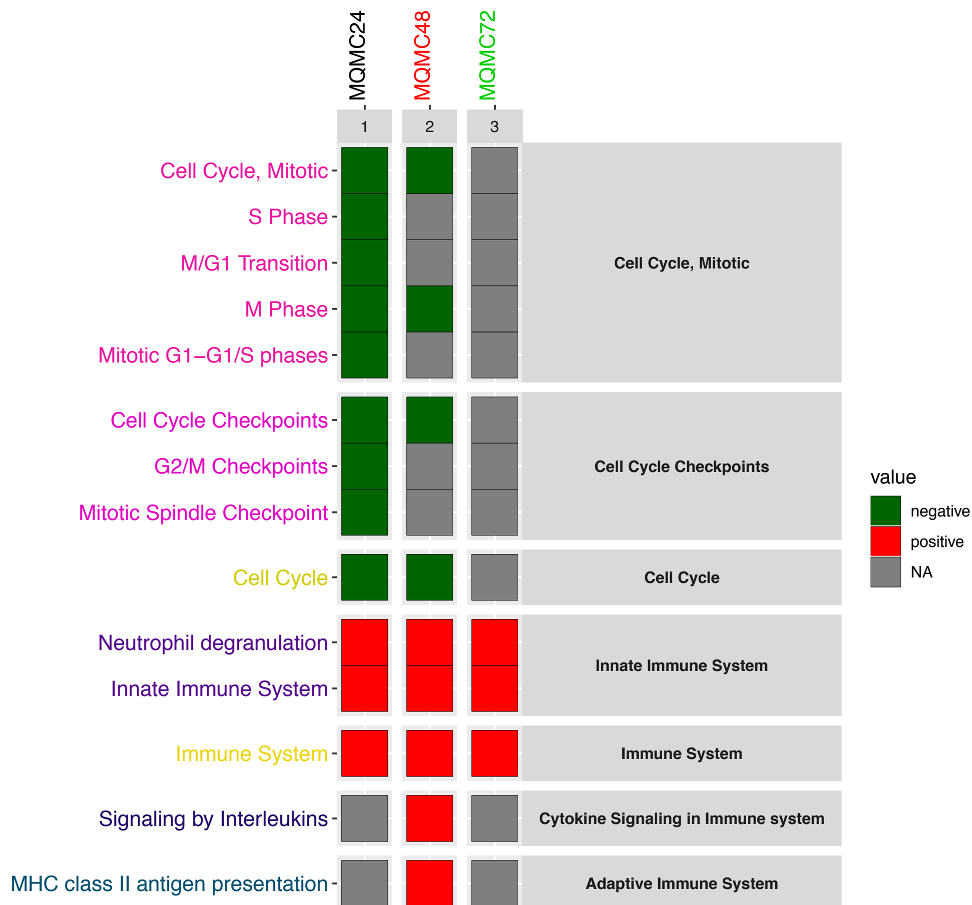
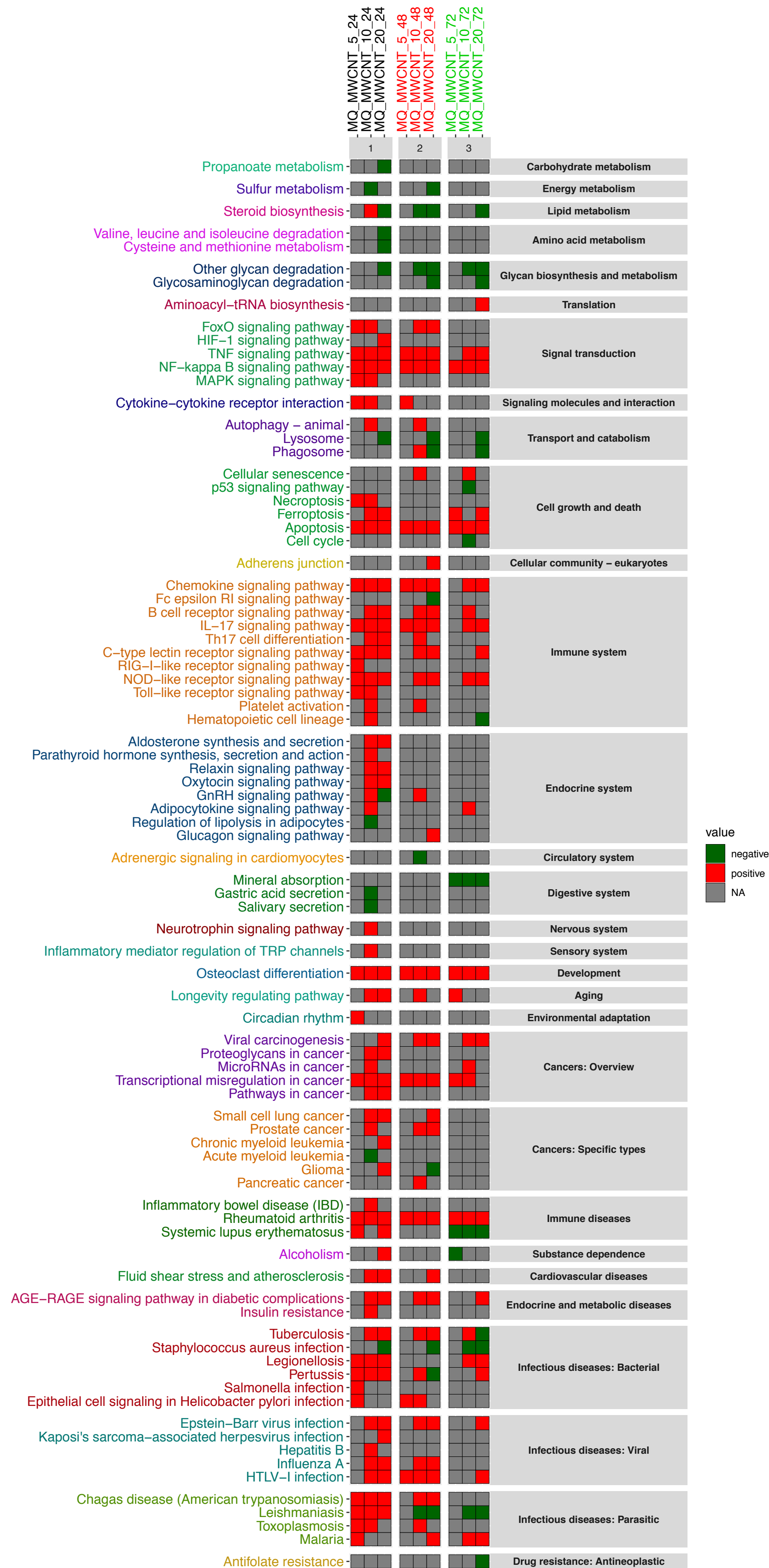
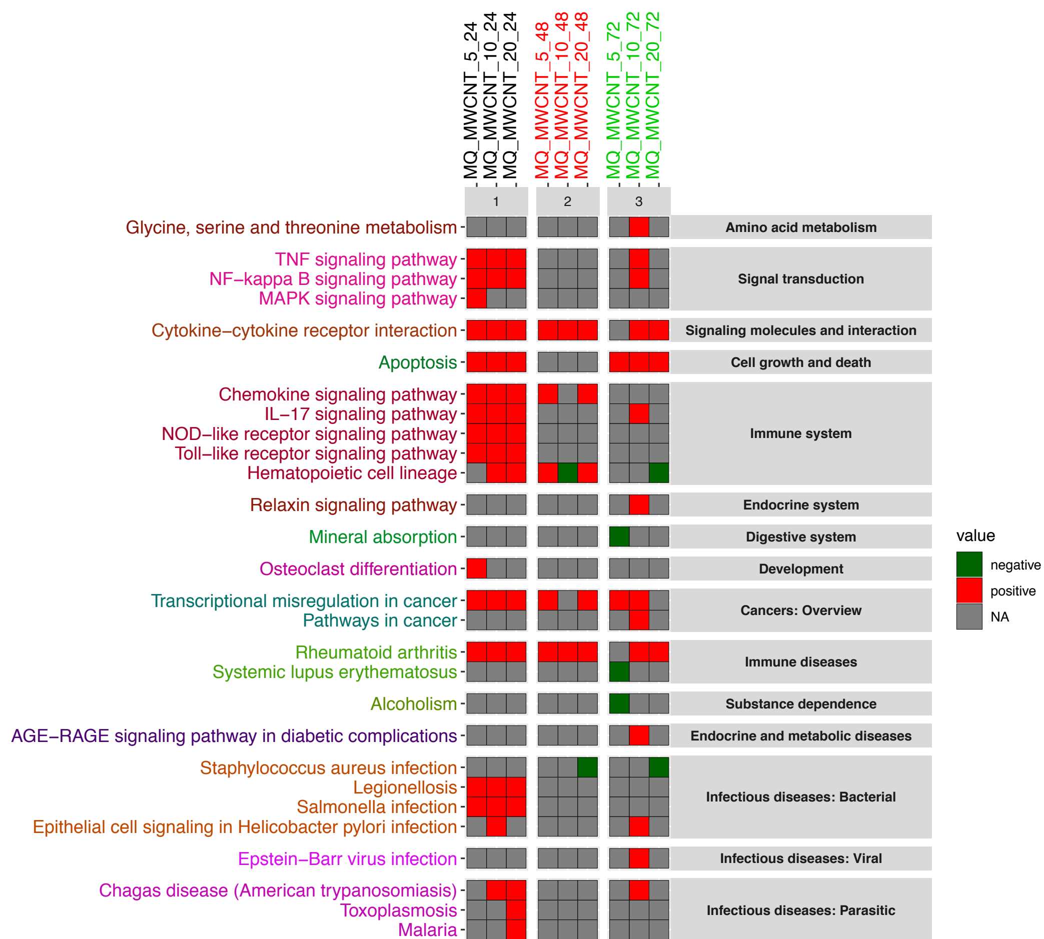
9 Appendices



Supplementary Figure 1. Enrichment map of Reactome pathways based on differentially expressed genes between PMA-differentiated macrophages and THP-1 monocytes at 24h, 48h and 72h. Colouring of the enrichment is based on the mean log2 FC of genes contributing to the enrichment.



Supplementary Figure 2. Total KEGG pathway enrichment map for differentially expressed genes in macrophages at 3 doses and 3 time points.



Supplementary Figure 3. KEGG pathway enrichment of top 100 upregulated and downregulated genes in macrophages upon MWCNT exposure.